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Di-Tripeptides and Oligopeptides Are Taken Up via Distinct Transport Mechanisms in *Lactococcus lactis*

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***Lactococcus lactis* ML3 possesses two different peptide transport systems of which the substrate size restriction and specificity have been determined. The first system is the earlier-described proton motive force-dependent di-tripeptide carrier (E. J. Smid, A. J. M. Driessen, and W. N. Konings, J. Bacteriol. 171:292–298, 1989). The second system is a metabolic energy-dependent oligopeptide transport system which transports peptides of four to at least six amino acid residues. The involvement of a specific oligopeptide transport system in the utilization of tetra-alanine and penta-alanine was established in a mutant of *L. lactis* MG1363 that was selected on the basis of resistance to toxic analogs of alanine and alanine-containing di- and tripeptides. This mutant is unable to transport alanine, dialanine, and trialanine but still shows uptake of tetra-alanine and penta-alanine. The oligopeptide transport system has a lower activity than the di-tripeptide transport system. Uptake of oligopeptides occurs in the absence of a proton motive force and is specifically inhibited by vanadate. The oligopeptide transport system is most likely driven by ATP or a related energy-rich, phosphorylated intermediate.**

Lactococci require, in addition to a carbohydrate source, nucleotides, vitamins (3), and amino acids (20) for growth. The actual number of amino acids required for growth is strain dependent. The amino acid requirement can be satisfied by free amino acids, peptides, and/or proteins (i.e., caseins). For degradation of proteins, lactococci possess an extracellular cell wall-bound proteinase and peptidases which act in concert to supply the cells with essential and growth-stimulating amino acids and small peptides (for reviews, see references 9 and 26). Transport of the casein-derived amino acids is mediated by several different amino acid transport systems (for a review, see reference 7).

In addition to amino acid uptake, lactococci can also satisfy their amino acid demand by uptake of peptides. One uptake system specific for di- and tripeptides has been investigated in a peptidase-free membrane vesicle system (23). In that study, alanyl-glutamate (Ala-Glu) was used as a model substrate. Accumulation of the dipeptide in membrane vesicles of *Lactococcus lactis* ML3 fused with liposomes containing beef heart cytochrome *c* oxidase was found to be driven by the electrical potential ($\Delta\psi$) and the chemical gradient of protons (ΔpH) across the membrane (23). Information about the specificity of this transport system is limited and is restricted to results obtained from competition experiments in which the rate of uptake of radioactively labelled peptides was estimated in the absence or presence of a few unlabelled peptides (10, 22, 23). Di-tripeptide transport was shown to be required for growth of *L. lactis* ML3 on the milk protein β-casein (25), indicating that one or more essential or growth-stimulating amino acids are released as di- or tripeptides during casein hydrolysis. The high proline content of β-casein (35 of 209 residues [21]) suggests that several essential and growth-stimulating casein-derived amino acids will be supplied to the cells as proline-containing peptides. The finding that especially proline-containing dipeptides are high-affinity substrates for the lactococcal di-tripeptide transport system is in agreement with this notion (24).

Information about the presence and properties of transport systems for oligopeptides (peptides containing four or more amino acid residues) is virtually lacking. Growth (10) and transport (22) studies with *L. lactis* have indicated that the size restriction for peptide utilization is four to five amino acid residues. In this study, the presence of an oligopeptide transport system in *L. lactis* was demonstrated in mutants devoid of alanine-glycine and/or di-tripeptide transport activity with tetra-alanine, penta-alanine, and hexa-alanine as substrates. The energetics of oligopeptide transport, the size restriction, and the specificity of the di-tripeptide and oligopeptide transport systems were analyzed.

MATERIALS AND METHODS

Culture conditions and growth media. Cultures of *L. lactis* subsp. *lactis* ML3 or MG1363 were maintained in 10% (wt/vol) skim milk containing 0.1% (wt/vol) tryptone (Difco, East Molesey, United Kingdom) and stored at −80°C. Mutants derived from these strains were stored in chemically defined medium (CDM) (15) supplemented with 10% (vol/vol) glycerol. For transport experiments, cells were cultivated in complex broth medium (MRS) (4), M17 (Difco), or CDM (all at pH 6.4). The medium was supplemented with separately sterilized glucose (0.5%, wt/vol), lactose (0.5%, wt/vol), or galactose (0.5%, wt/vol) in combination with 25 mM arginine to induce the arginine deiminase (ADI) pathway. Cells were incubated at 30°C.

Isolation of mutants resistant to toxic amino acid and peptide analogs. Spontaneous L-β-chloroalanine-resistant mutants of *L. lactis* ML3 were isolated with a frequency of $6 \cdot 10^{-8}$ on 1% agar plates containing CDM from which alanine and glycine were omitted and which was supple-

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mented with 50 μM L-β-chloroalanine. The clones were examined for transport of alanine and glycine. In *L. lactis* ML3, both amino acids are transported by a common transport system (5). The degree of reduction of alanine and glycine uptake in these mutants varied from 18 to 100%, indicating different defects in the lactococcal alanine-glycine transport system (see Table 1). Spontaneous alanyl-alanyl-β-chloroalanine-resistant mutants of *L. lactis* ML3 were isolated from plates containing a 0.25 mM concentration of the toxic tripeptide (strain MLDT2). An alanyl-β-chloroalanine-resistant mutant of *L. lactis* MG1363 (MGDT1) defective in alanyl[^{14}C]glutamate uptake was subsequently grown on plates containing 50 μM β-chloroalanine. Several spontaneous mutants which were resistant to β-chloroalanine and alanyl-β-chloroalanine were isolated with a mutation frequency of $3 \cdot 10^{-7}$ (see Table 1). The double mutants, defective in both alanine and di-tripeptide uptake, are referred to as MGAD; MGAD4 was used for most studies (see Results).

Transport assays. Prior to transport, cells were washed twice with 100 mM potassium phosphate, pH 6.5, and resuspended to a final A_{660} of approximately 25. To de-energize the cells, cell suspensions were incubated with 10 mM 2-deoxyglucose for 20 min at 30°C. This procedure results in depletion of the intracellular amino acid pool (19). Cells were subsequently washed twice with potassium phosphate, pH 6.5. In experiments in which vanadate or arsenate was used, the buffer was replaced by 100 mM potassium–2-(*N*-morpholino) ethanesulfonic acid (MES). The pH dependency of transport was determined in 30 mM MES–30 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES)–30 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–5 mM $MgSO_4$. The buffers were adjusted to the desired pH with KOH. All transport assays were performed at 30°C. Transport of radioactively labelled compounds in intact cells was done as previously described (23). Transport of unlabelled peptides was monitored by determining the intracellular concentration of the corresponding amino acids by means of reversed-phase high-performance liquid chromatography analysis. For this purpose, a new method was developed which essentially combines the filtration method used in transport assays of radioactively labelled compounds (23) and extraction of amino acids and peptides from cells by perchloric acid treatment. Following incubation in the presence of substrates, cells (1 ml; A_{660}, 1 to 5) were collected on 0.45 μm-pore-size cellulose acetate filters (Schleicher & Schuell GmbH, Dassel, Germany) by using a manifold filtration apparatus (Hoefer, San Francisco, Calif.) with vacuum applied by a Divac 2.4 L pump (Leybold AG, Cologne, Germany). The cells were washed three times with ice-cold potassium phosphate (100 mM, pH 6.5). The filter was subsequently transferred to a vial (20 ml; Packard, Canberra Industries, Meriden, Conn.), which contained 300 μl of 5% (vol/vol) perchloric acid and 10 mM Na-EDTA. After 30 min of incubation, 110 μl of acidic cell extract was pipetted into an Eppendorf tube containing 100 μl of 1 M KOH–1 M $KHCO_3$ to adjust the pH to 9.5. Samples were subsequently stored at −20°C. Control experiments showed that the cellulose acetate filters were not hydrolyzed during incubation in perchloric acid. Amino acids and peptides were analyzed after derivatization with dansyl chloride, by using essentially the methods described by Tapuhi et al. (27) and Wiedmeier et al. (29). The dansylated, neutralized cell extracts were separated by high-performance liquid chromatography as described previously (19).

ATP measurements. Samples were taken, and cells were extracted with 5% perchloric acid and 10 mM Na-EDTA as described above. The extract was neutralized with 1 M KOH–1 M $KHCO_3$ and diluted 40-fold into 40 mM Tris-acetate, pH 7.6. ATP concentrations were subsequently determined by the firefly luciferase assay essentially as described by Lundin and Thore (12).

Miscellaneous. Protein was determined by the method of Lowry et al. (11), with bovine serum albumin as the standard. The glycolytic and ADI pathway activities and the membrane potential ($\Delta\psi$) were determined as described previously (8, 19). Growth experiments were performed with enzyme-linked immunosorbent assay plates, and specific growth rates were estimated from A_{660} measurements with a Thermo Max apparatus (Molecular Devices, Palo Alto, Calif.).

Chemicals. L-Alanyl-L-[^{14}C]glutamate (57 mCi/mmol) was synthesized as described previously (23). L-Alanyl-L-alanyl-β-chloro-L-alanine and L-alanyl-β-chloro-L-alanine were prepared by solid-phase peptide synthesis (1). The required 9-fluoroenylmethoxycarbonyl-β-chloro-L-alanine pentafluorophenyl ester was prepared by the general method of Kisfaludy and Schön (6). All other peptides were obtained from Sigma Chemical Company, St. Louis, Mo., or Bachem Feinchemikalien AG, Bubendorf, Switzerland. Unless indicated otherwise, all peptides and amino acids were in the L configuration. All other chemicals were of reagent grade and were obtained from commercial sources.

RESULTS

Isolation and characterization of transport mutants. In a previous study, it was shown that dipeptide transport-deficient mutants of *L. lactis* subsp. *lactis* ML3 could be isolated on media containing the toxic dipeptide alanyl-β-chloroalanine (e.g., MLDT1) (25). Also, the tripeptide alanyl-alanyl-β-chloroalanine appeared to be toxic for wild-type *L. lactis* ML3 (data not shown). The alanyl-β-chloroalanine-resistant mutants (e.g., MLDT1) were found to be resistant to high concentrations (up to 1 mM) of the toxic tripeptide (data not shown). The alanyl-alanyl-β-chloroalanine mutants (e.g., MLDT2) displayed a highly reduced capacity for uptake of the dipeptide Ala-Glu and were, with respect to peptide transport, phenotypically similar to the alanyl-β-chloroalanine-resistant mutants (Table 1).

The β-chloroalanine- and alanyl-β-chloroalanine-resistant double mutants (MGAD1-MGAD6) were defective in L-Ala–Glu uptake but varied with respect to the ability to transport alanine (Table 1). The spontaneous β-chloroalanine-resistant mutants must have arisen through decreased alanine transport activity and/or altered activity of one or more biosynthetic enzymes which prevent incorporation of β-chloroalanine. The mutant with the lowest initial rate of alanine uptake and the lowest level of alanine accumulation, designated MGAD4 (AlaT$^-$ DtpT$^-$), was also tested for growth on CDM with alanine, dialanine, trialanine, tetra-alanine, or penta-alanine and on CDM without further additions (Fig. 1). This mutant grew slowly on alanine, dialanine, and trialanine, with rates comparable to those of wild-type cells in an alanine-deficient growth medium, whereas growth on tetra-alanine and penta-alanine was comparable to that of the wild type. In the absence of alanine and alanine-containing peptides, growth of the MGAD4 (AlaT$^-$ DtpT$^-$) mutant strain displayed a lag phase whereas the wild-type strain did not. This difference is most likely related to some residual alanine (<20 μM) that is transferred from the preculture medium to the fresh growth medium upon inoculation. Wild-type cells

TABLE 1. Isolated mutants of *L. lactis* ML3 and MG1363 and their characterization with respect to alanine, di-tripeptide, and oligopeptide transport[a]

Strain tested	Parent strain	Resistance[b]	Transport activity (%)		
			Ala	Ala_2	Ala_4
ML3[c]			100	100	100
MLAT1	ML3	β-Chloroalanine	1	102	95
MLAT2	ML3	β-Chloroalanine	82	ND[d]	ND
MLAT3	ML3	β-Chloroalanine	10	ND	ND
MLDT1	ML3	Alanyl-β-chloroalanine	135	4	220
MLDT2	ML3	Alanyl-alanyl-β-chloroalanine	ND	6	ND
MG1363[e]			100	100	100
MGDT1	MG1363	Alanyl-β-chloroalanine	100	<5	ND
MGAD1	MGDT1	β-Chloroalanine	33	<5	ND
MGAD2	MGDT1	β-Chloroalanine	13	<5	ND
MGAD3	MGDT1	β-Chloroalanine	30	<5	ND
MGAD4	MGDT1	β-Chloroalanine	8	<5	85
MGAD5	MGDT1	β-Chloroalanine	30	<5	ND
MGAD6	MGDT1	β-Chloroalanine	18	<5	ND

[a] Mutants were isolated by selection for β-chloroalanine, alanyl-β-chloroalanine, or alanyl-alanyl-β-chloroalanine resistance, as indicated. Di-tripeptide transport activity was inferred from uptake of dialanine (Ala_2), and oligopeptide transport activity was inferred from uptake of tetra-alanine (Ala_4), as described in Materials and Methods. The 100% activities of alanine, dialanine, and tetra-alanine transport correspond to 20, 41, and 3 nmol/min/mg of protein, respectively.

[b] Mutants were selected on the basis of resistance to β-chloroalanine or alanine-containing peptides.

[c] Wild-type strain.

[d] ND, not done.

[e] Plasmid-free derivative of ML3.

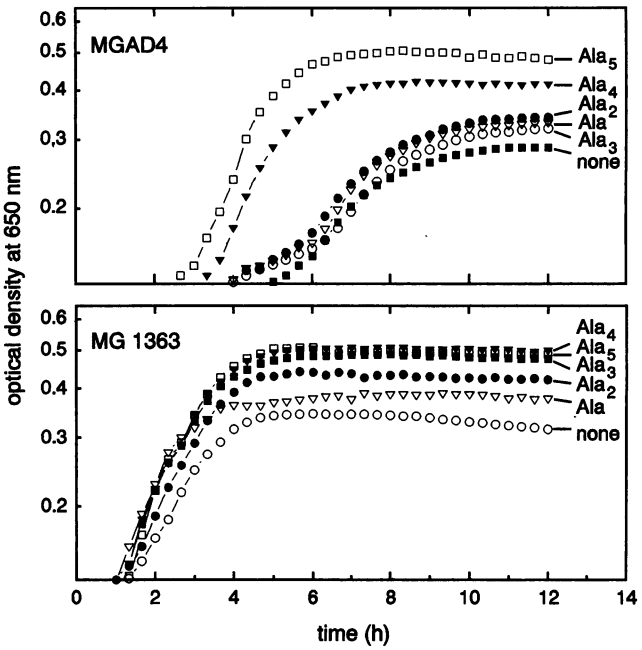

FIG. 1. Growth curves for wild-type strain MG1363 and mutant strain MGAD4 ($AlaT^-$ $DtpT^-$) grown on CDM with or without alanine or alanine-containing peptides. Prior to the growth experiments, *L. lactis* MG1363 was cultured on CDM containing 1.1 mM alanine and 2.3 mM glycine and *L. lactis* MGAD4 ($AlaT^-$ $DtpT^-$) was cultured on CDM without alanine-glycine and supplemented with β-chloroalanine (50 μM) and alanyl-β-chloroalanine (250 μM). Cells were then diluted (50-fold) in CDM without alanine to a final A_{660} of about 0.05. Alanine or alanine-containing peptides (1 mM) were added as indicated.

are able to utilize this alanine, and this may have shortened the lag time prior to growth. Analysis of peptide uptake in MGAD4 ($AlaT^-$ $DtpT^-$) is in agreement with the findings with respect to growth (see below). MGAD4 ($AlaT^-$ $DtpT^-$) was further characterized to exclude the possibility that the reduced transport activities are the result of defects in the generation of metabolic energy. The glycolytic and ADI pathway activities of these cells, estimated from the rates of product formation (8), were found to be comparable to those of wild-type cells. Furthermore, MGAD4 ($AlaT^-$ $DtpT^-$) is capable of generating a Δp and ATP to wild-type levels (data not shown).

Size restriction of the dipeptide transport system. The peptide size restriction range of the recently described dipeptide transport system of *L. lactis* ML3 (23) was studied in glycolyzing cells by measuring the uptake of radioactively labelled Ala-Glu in the presence of a 500-fold excess of unlabelled peptides. Figure 2A shows that the presence of excess alanine did not significantly influence uptake of radioactively labelled Ala-Glu, whereas profound inhibition of Ala-Glu uptake was observed in the presence of excess alanyl-alanine, alanyl-alanyl-alanine, and histidyl-glycyl-glycine. On the other hand, oligopeptides such as tetra-alanine, penta-alanine, hexa-alanine, Ala-Gly-Ser-Glu, and Val-Gly-Asp-Glu did not inhibit uptake of Ala-Glu significantly (Fig. 2B). These competition experiments suggest that the dipeptide transport system has affinity for both di- and tripeptides but not for oligopeptides composed of more than three amino acids.

Substrate specificity of the di-tripeptide transport system of *L. lactis*. Different di- and tripeptides inhibit Ala-Glu uptake (Fig. 2A). Furthermore, alanyl-β-chloroalanine- and alanyl-alanyl-β-chloroalanine-resistant mutants were phenotypically indistinguishable from each other with respect to peptide uptake (data not shown). To demonstrate that di- and tripeptide transport actually occurs, the uptake of several peptides was studied in the *L. lactis* ML3 wild type and in dipeptide transport-deficient mutant MLDT1. Table 2 shows that both X-pro and pro-X dipeptides were taken up by the *L. lactis* ML3 wild type. Uptake of both types of proline-containing peptides was severely reduced in the MLDT1 ($DtpT^-$) strain. Comparable findings were obtained with zwitterionic dipeptides (Met-Met and Leu-Leu) and acidic dipeptides (Glu-Val and Ala-Glu). The latter finding indicates that the position of the acidic residue in the dipeptide is not crucial. Transport of arginine-containing dipeptides (Ile-Arg, Arg-Ile, and Arg-Arg) was not detected (Table 2). Also, dipeptides with amino acids in the D configuration (D-Ala–D-Glu) and dipeptides with an elongated backbone (β-Ala–DL-Leu) were not taken up by *L. lactis* ML3.

The tripeptides Gly-Ser-Ala and Gly-Pro-Ala were taken up by *L. lactis* ML3 at a much lower rate than was trialanine. Uptake of other tripeptides, such as Pro-His-Val and Pro-Gly-Gly, could hardly be detected. The alanyl-β-chloroalanine-resistant mutant displayed reduced uptake of tripeptides, which is in accordance with the results of the competition experiments (Fig. 2) and with the properties of the alanyl-alanyl-β-chloroalanyl-resistant mutant (MLDT2).

Utilization of oligopeptides by *L. lactis*. Certain oligopeptides can be utilized by lactococci as organic nitrogen sources (10). To demonstrate the involvement of a specific

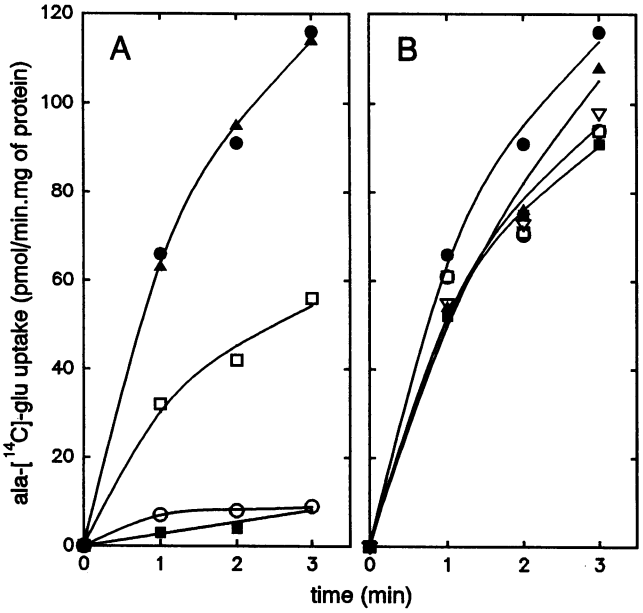

FIG. 2. Alanyl[^{14}C]glutamate uptake in lactose-metabolizing cells of *L. lactis* ML3 in the presence or absence of 1 mM unlabelled substrate. Uptake of alanyl[^{14}C]glutamate in the presence of unlabelled alanine (▲), Ala-Ala (■), Ala-Ala-Ala (□), and His-Gly-Gly (○) is shown in panel A. Panel B shows energized uptake in the presence of tetra-alanine (■), penta-alanine (○), hexa-alanine (□), Ala-Gly-Ser-Glu (▽), and Val-Gly-Asp-Glu (▲). Uptake of alanyl [^{14}C]glutamate in the absence of unlabelled substrate (●) is presented in both panels. The uptake assay was started by addition of 2 μM alanyl[^{14}C]glutamate.

TABLE 2. Uptake of different di-, tri-, and oligopeptides by glycolyzing cells of *L. lactis* wild-type ML3 and mutant MLDT1 (DtpT$^-$)[a]

Peptide (concn [mM])	Uptake rate (nmol/min/mg of protein)[b]	
	Wild type	MLDT1
Leu-Pro* (0.95)	95	17
Met-Pro* (0.95)	87	5
Pro*-Met (0.95)	51	2
Met*-Met* (0.48)	50	6
Leu-Leu[c] (0.48)	44	9.1
Ala-Glu[c] (0.85)	17	0.7
Glu-Val* (0.48)	11	1.2
Ile-Arg (0.48)	<0.1	<0.1
Arg-Ile (0.48)	<0.1	<0.1
Arg-Arg (0.48)	<0.1	<0.1
D-Ala-D-Glu (0.48)	<0.1	ND[d]
β-Ala-DL-Leu (0.48)	<0.1	ND
Ala*-Ala*-Ala* (0.48)	60	12
Gly-Ser-Ala* (0.48)	13	ND
Gly-Pro*-Ala (0.48)	6.4	1.1
Pro*-His-Val (0.48)	1.0	<0.1
Pro-Gly-Gly (0.48)	<0.1	ND
Tetra-alanine (0.48)	2.6	2.9

[a] Reference 25.
[b] Peptide uptake rates were determined from the time-dependent increase of the intracellular amino acid pools as described in Materials and Methods. The amino acids marked with asterisks were used to determine the rates shown.
[c] Uptake was measured with radioactively labelled dipeptides.
[d] ND, not done.

transport system in the uptake of oligopeptides, it is essential to exclude the possibility that the peptides are hydrolyzed by extracellularly located peptidases and that the apparent uptake is due to amino acid and/or di-tripeptide transport activities. For this purpose, a mutant deficient in di-tripeptide transport (MLDT1) and one deficient in alanine-glycine transport (MLAT1) were used. Glycolyzing cells of the wild type, the di-tripeptide transport mutant (MLDT1), and the alanine-glycine transport mutant (MLAT1) were incubated for 10 min with either 0.5 mM alanine or di-, tri-, tetra-, penta-, or hexa-alanine (Fig. 3). The increase of the internal alanine pool was taken as an estimate for uptake of the different substrates. Assuming that the intracellular peptidase activity is not rate limiting in peptide utilization (28), the uptake rates of the different alanyl peptides can be calculated by dividing the observed rate of increase of the alanine pool by the number of alanine residues in the peptides.

Rapid alanine uptake occurred in the wild-type and MLDT1 (DtpT$^-$) strains but not in the MLAT1 strain (AlaT$^-$) (Fig. 3A). The increased rate of alanine uptake in the di-tripeptide transport-deficient mutant (MLDT1) in comparison with the wild-type strain is in accordance with previous findings that expression of the di-tripeptide transport system and growth in peptide-containing media affect the expression of various amino acid transport systems (15, 25). As judged from the increase of the intracellular alanine pools, both the wild-type and MLAT1 (AlaT$^-$) strains have comparable rates of di- and trialanine uptake (Fig. 3B and C). The MLDT1 strain exhibited highly reduced di- and trialanine uptake rates (Fig. 3B and C). When glycolyzing cells of *L. lactis* ML3 (wild type) were incubated with tetra-, penta-, or hexa-alanine, an increase of the intracellular alanine pool was observed which was slower than with di- or trialanine as the substrate (Fig. 3D, E, and F). It is of interest that the increase of intracellular alanine in the MLAT1 and MLDT1 strains was, with all three oligopeptides, the same as or higher than that in the wild type, suggesting that expression of the oligopeptide transport system in the mutant strains was somewhat increased. The observed tetra-, penta, and hexa-alanine uptake rates of *L. lactis* ML3 (at an external peptide concentration of 0.5 mM) were 2.3, 8.0, and 2.3 nmol/min/mg of protein, respectively.

Although dipeptide transport was not significantly inhibited by a 500-fold molar excess of tetra-, penta-, or hexa-alanine (Fig. 2B), uptake of these three oligopeptides was completely abolished by a 20-fold excess of Leu-Leu (data not shown), indicating that the oligopeptide transport system either binds or translocates dipeptides to some extent.

To demonstrate unequivocally that uptake of peptides larger than three residues is the result of a specific transport system, oligopeptide transport was also analyzed in the MGAD4 (AlaT$^-$ DtpT$^-$) double mutant. The double mutant transported tetra-alanine and penta-alanine with rates similar to those of the wild type, whereas alanine, di-alanine, and tri-alanine were not taken up (Fig. 4).

Energetics of oligopeptide transport. To characterize oligopeptide transport in *L. lactis* ML3 in more detail, tetra-alanine was chosen as the model substrate for further studies. With no energy source, no significant uptake of the peptide was detected. Addition of a fermentable sugar (glucose) resulted in a high rate of tetra-alanine uptake (Fig. 5). At pH 7.5, when the external and internal pHs are similar (13), tetra-alanine uptake in *L. lactis* ML3 was slightly reduced by addition of valinomycin, a potassium ionophore

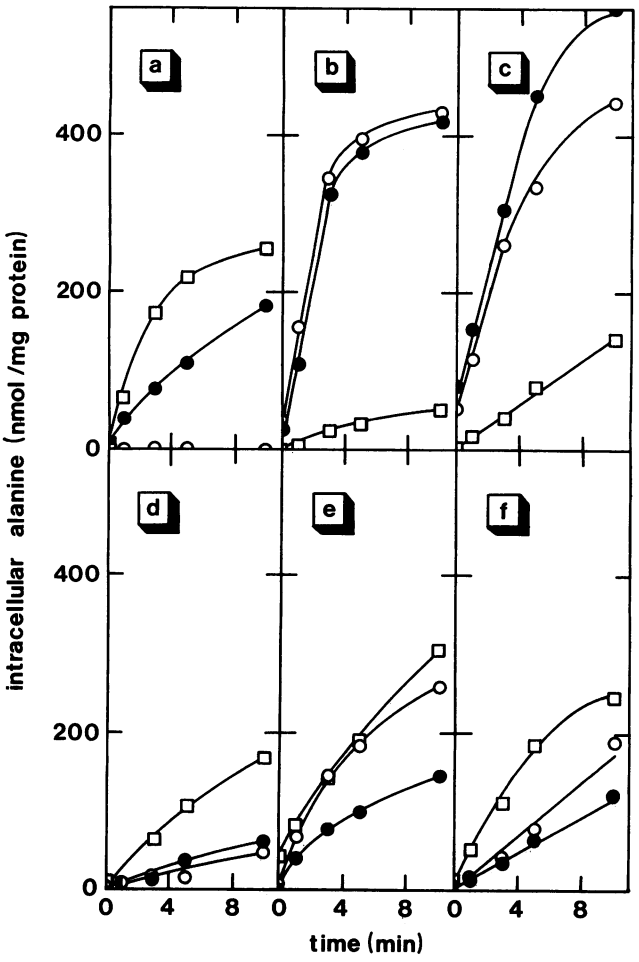

FIG. 3. Time course of the internal alanine pool in glycolyzing cells of *L. lactis* ML3 in response to addition of alanine (A), dialanine (B), trialanine (C), tetra-alanine (D), penta-alanine (E) or hexa-alanine (F). Pool measurements were performed with strains ML3 (●), MLAT1 (AlaT$^-$) (○), and MLDT1 (DtpT$^-$) (□). Cells were grown in complex broth medium (MRS) supplemented with 0.5% lactose. Prior to the transport assays, cells were de-energized as described in Materials and Methods. The cell suspensions were pre-energized for 2 min with 0.4% lactose in 100 mM MES (pH 6.5) before the transport assay was started by addition of the substrates at 0.5 mM. The alanine pools were quantified as described in Materials and Methods.

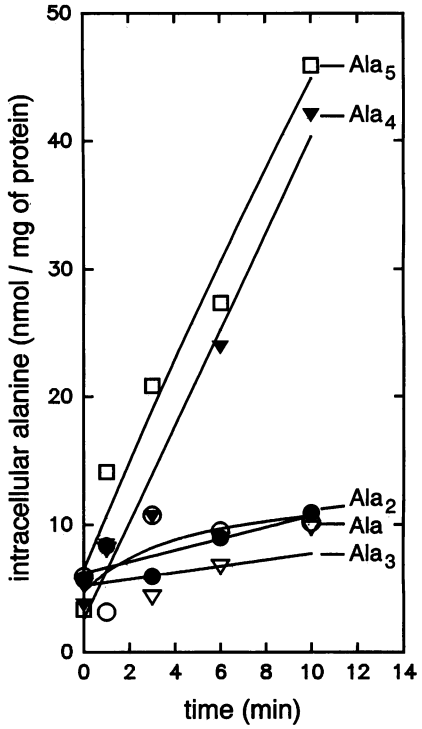

FIG. 4. Time course of the internal alanine pool in glycolyzing cells of *L. lactis* MGAD4 (AlaT$^-$ DtpT$^-$) in response to addition of alanine and alanine-containing peptides. Cells were grown in CDM supplemented with 0.5% (wt/vol) glucose. Prior to the transport assays, cells were de-energized as described in Materials and Methods. The cell suspensions were pre-energized for 5 min with 0.5% (wt/vol) glucose in 100 mM potassium phosphate, pH 6.5, before the uptake assay was started by addition of the substrate at 0.5 mM. Alanine pools were determined as described in Materials and Methods.

which dissipates the membrane potential in the presence of K^+ (Fig. 5A). The potassium-proton ionophore nigericin had no effect on tetra-alanine uptake. Addition of both ionophores, which dissipated the Δp completely (data not shown), inhibited tetra-alanine uptake partially. The intracellular ATP pool was lowered by about 30% in the presence of valinomycin plus nigericin (data not shown). Uptake of tetra-alanine by strain MGAD4 (AlaT$^-$ DtpT$^-$) was not affected by valinomycin or nigericin. Addition of both ionophores caused some inhibition of tetra-alanine uptake in MGAD4 (AlaT$^-$ DtpT$^-$), but the effect was significantly less than that in *L. lactis* ML3 (Fig. 6B). These experiments show that tetra-alanine transport can proceed in the absence of electrochemical gradients for protons and potassium ions. To investigate further the nature of the energy source of oligopeptide transport, the effects of ATPase inhibitors on tetra-alanine uptake were studied. Since ATP production by the glycolytic pathway is affected by vanadate and arsenate, the ADI pathway was used for generation of metabolic energy. In the ADI pathway, arginine is converted into ornithine, ammonia, and carbon dioxide, which yields 1 mol of ATP per mol of arginine metabolized. ADI pathway activity (ammonia production) and the resulting ATP production were hardly affected by vanadate and arsenate (each at 0.5 mM [final concentration]). Addition of arginine led to rapid accumulation of alanine in these cells (Fig. 6A). Neither vanadate nor arsenate influenced alanine uptake, which was expected, since alanine transport is driven by the proton motive force and is not affected by ATP directly (5). In contrast, arginine-energized tetra-alanine uptake was completely blocked by vanadate while arsenate had no effect (Fig. 6B). Parallel to the transport assays, samples were taken for determination of internal ATP concentrations (Fig. 7). After addition of arginine to de-energized cells, the internal ATP concentration increased from 0.5 to 1.8 mM. Addition of vanadate or arsenate did not significantly influence ATP production (1.5 to 1.6 mM). These findings indicate that inhibition of tetra-alanine transport by vanadate must be due to inhibition of the transport system directly.

Induction and activity of the transport system. The activity of the oligopeptide transport system varied with the growth conditions. An approximately twofold increase in activity was observed when complex medium M17 instead of CDM

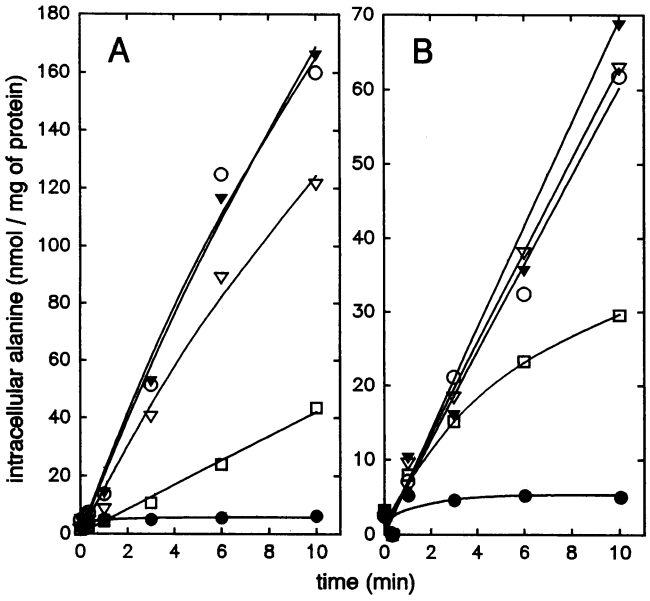

FIG. 5. Effects of ionophores on tetra-alanine uptake in *L. lactis* ML3 (A) and MGAD4 (AlaT$^-$ DtpT$^-$) (B). *L. lactis* ML3 was grown on M17, and MGAD4 (AlaT$^-$ DtpT$^-$) was grown on CDM, both supplemented with 0.5% (wt/vol) glucose. Prior to the transport assays, cells were de-energized as described in Materials and Methods. For the transport assay, cells were incubated in 100 mM potassium phosphate, pH 7.5, containing either glucose (○) or no energy source (●), glucose plus valinomycin (▽), or glucose plus nigericin (▼). Valinomycin and nigericin were added to final concentrations of 0.8 and 0.4 μM, respectively. The same concentrations were employed when both ionophores were added (□). The uptake assay was started, after 5 min of pre-energization in the presence of 0.4% glucose, by adding 1 mM penta-alanine. The transport activity of *L. lactis* ML3 was higher than that of MGAD4 (AlaT$^-$ DtpT$^-$) because of a higher level of expression of the transport system upon growth in a complex medium.

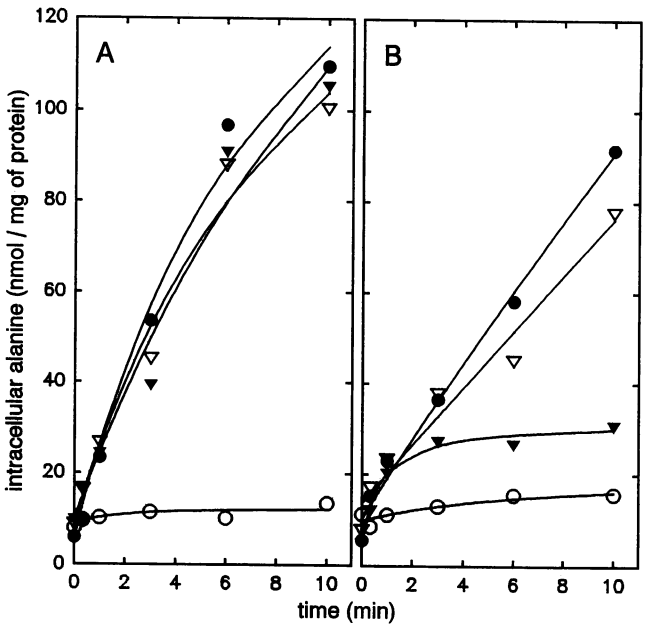

FIG. 6. Effects of vanadate and arsenate on uptake of alanine (A) and tetra-alanine (B) in arginine-energized cells of *L. lactis* ML3. To induce the ADI pathway, cells were grown in CDM supplemented with 0.5% galactose and 25 mM arginine. Prior to the transport assays, cells were de-energized as described in Materials and Methods. The cell suspensions were pre-energized for 7 min with 25 mM arginine in 100 mM MES, pH 6.5 (●), before the uptake assay was started by addition of the substrate at 1.0 mM. Vanadate (▼) and arsenate (▽) were each added at the beginning of the pre-energization period to a final concentration of 0.5 mM. ○, uptake in the absence of an energy source. Alanine pools were determined as described in Materials and Methods.

was used to culture the cells (Fig. 5, legend). Uptake rates of tetra-alanine were also dependent on the energy source. The rate of uptake and accumulation levels were at least twice as high with glucose as with arginine as the energy source. This could be related to the difference in ATP concentrations inside the cell, i.e., 2.65 mM with glucose and 1.8 mM with arginine as the source of metabolic energy.

pH dependence of peptide transport. The external pH dependence of Ala-Glu and penta-alanine uptake in glycolyzing cells of *L. lactis* subsp. *lactis* ML3 was measured (data not shown). Ala-Glu uptake showed a broad optimum from pH 5 to pH 7.5. Below pH 4, no significant uptake was observed. The highest penta-alanine uptake rates were observed between pHs 6 and 7.5. At every pH value, the rate of penta-alanine uptake was lower than the rate of Ala-Glu uptake. The broad pH optima of the di-tripeptide and oligopeptide transport systems indicate that both systems are operative in the physiological pH range (15).

DISCUSSION

This report describes the presence of two different peptide transport systems in *L. lactis*. Uptake studies with *L. lactis* wild-type ML3 and MLDT1 (DtpT$^-$) (Table 2) and substrate competition experiments (Fig. 2) indicated that one (major) system preferentially translocates dipeptides and, to a lesser extent, tripeptides. The di-tripeptide uptake system transports a variety of structurally different di- and tripeptides, including neutral and acidic, but not basic, ones. The conclusion that di- and tripeptides share the same transport system is supported by the finding that Ala-Glu transport was reduced to the same extent in both alanyl-alanyl-β-chloroalanine (a toxic tripeptide)- and alanyl-β-chloroalanine (a toxic dipeptide)-resistant mutants of *L. lactis* ML3 (Table 1). This peptide transport system, designated the lactococcal di-tripeptide transport system (DtpT), exhibits no affinity for peptides composed of more than three amino acid residues. A second peptide transport system, which has a relatively low level of activity, translocates oligopeptides. Specific oligopeptide transport was observed in alanine and di-tripeptide transport-deficient mutants (Fig. 3 and 4). These experiments exclude the involvement of an extracellular peptidase (e.g., aminopeptidase) in combination with an amino acid transport system (in this case, the alanine-glycine transport system) and/or the di-tripeptide transport system in the utilization of the tested oligopeptides.

The finding that tetra-, penta-, and hexa-alanine uptake was completely inhibited by excess Leu-Leu is in accordance with the findings of Rice et al. (22), which could be due to competition of di-tripeptides and oligopeptides for the same binding site. We cannot exclude the possibility that the oligopeptide transport system is actually able to translocate dipeptides. Dipeptide uptake by the oligopeptide transport system would also offer an explanation for residual dipeptide uptake by alanyl-β-chloroalanine-resistant mutants (Table 2

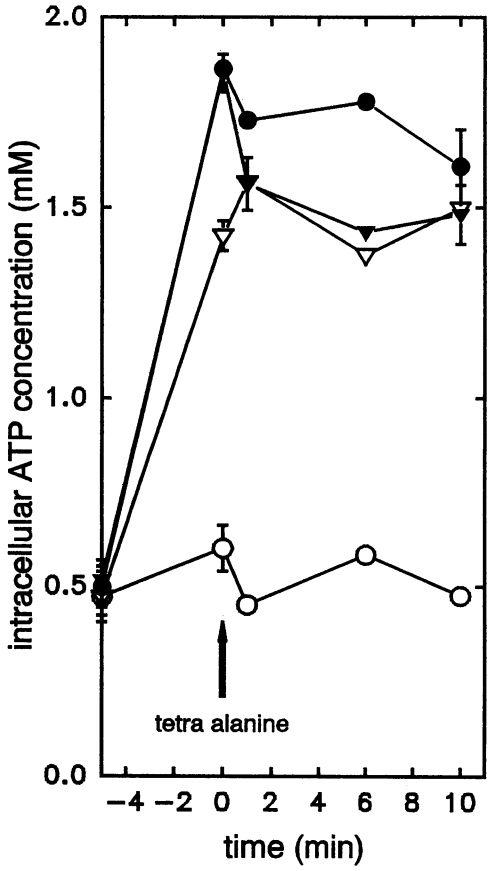

FIG. 7. Effects of vanadate and arsenate on the ATP concentration in arginine-energized cells of *L. lactis* ML3. ATP concentrations were determined in parallel with the uptake experiments (Fig. 6). For experimental conditions and symbols, see the legend to Fig. 6. The arrow indicates addition of 1 mM tetra-alanine.

and Fig. 3). Kinetic experiments with Leu-Leu, Gly-Leu (22), Ala-Glu (23), and Leu-Pro (26) uptake in *L. lactis* subsp. *cremoris* Wg2, *L. lactis* subsp. *lactis* C10, and *L. lactis* ML3, respectively, never revealed biphasic kinetics. However, this could be due to the low level of activity of the oligopeptide transport system, which makes it difficult to resolve a second kinetic component.

Although the rate of tripeptide uptake in *L. lactis* ML3 is generally lower than the rate of dipeptide uptake, the net rates of alanine intake into cells were comparable for both peptides (Fig. 3). The net rate of alanine intake by the oligopeptide transport system was, however, significantly lower. Uptake of tetra-alanine was found to be dependent on the presence of an energy source. At pH 7.5, only partial inhibition of tetra-alanine uptake was observed upon addition of valinomycin plus nigericin. In the presence of nigericin (with or without valinomycin) and at an external pH of 7.5, the internal pH is lowered to about 6.8 (14). Such a lowering of the internal pH affects the activities of various phosphate bond-driven transport systems in lactococci, as has been shown for uptake of glutamate-glutamine (14, 18), phosphate (17), and asparagine (13). Since inhibition of tetra-alanine uptake becomes pronounced only when nigericin and valinomycin are present, it seems unlikely that the internal pH is a major factor in controlling the activity of the oligopeptide transporter. During the generation of a pH gradient across the cytoplasmic membrane, potassium ions accumulate as a consequence of proton extrusion and preservation of electroneutrality (2). In the presence of valinomycin plus nigericin, the internal potassium pool is lowered effectively from 800 to ≤100 mM (14). It is possible that the intracellular concentration of potassium ions or another related parameter (e.g., turgor pressure) affects the activity of the oligopeptide transport system, which causes inhibition in the presence of both ionophores. Moreover, upon entry of tetra-alanine into the cells, the oligopeptide is rapidly hydrolyzed and efflux of alanine down its concentration gradient may occur. This is most pronounced under conditions in which valinomycin and nigericin are present, i.e., when the Δp is totally dissipated. Notice that alanine transport is driven by a Δp and that the transport activity increases with decreasing internal pH (5). Alanine efflux leads to underestimation of the apparent uptake of tetra-alanine. In strain MGAD4 ($AlaT^-$ $DtpT^-$), the effect of valinomycin plus nigericin on tetra-alanine uptake, on the basis of the internal alanine concentration, is indeed smaller than in the wild-type ($AlaT^+$) strain (Fig. 5). Finally, the 30% reduction of the ATP pool upon addition of valinomycin plus nigericin could also be reflected in the somewhat lower (apparent) uptake of tetra-alanine.

In arginine-energized cells, Δp-driven alanine transport is not affected by addition of the ATPase inhibitor vanadate, because the Δp-generating F_0F_1 ATPase of *L. lactis* is insensitive to vanadate. In contrast, tetra-alanine transport can be inhibited by vanadate, which indicates that this inhibitor must act on the transport system itself. ATP-driven translocators form a broad class of transport systems that junction in the uptake and excretion of various solutes (for a review, see reference 16). Many of these ATP-driven transport systems are sensitive to vanadate. On the basis of inhibition by vanadate and the significant oligopeptide transport activity under conditions in which the electrochemical proton gradient is absent, it is concluded that oligopeptide transport functions independently of the Δp and that the driving force is supplied by ATP or related phosphate bond energy. In conclusion, by isolating mutants defective in di-tripeptide and alanine transport, the presence of a peptide transport system specific for peptides of four to at least six amino acid residues was demonstrated. In addition to a distinct substrate specificity, the oligopeptide transport system differs from the di-tripeptide transport system in its energy requirement for transport, i.e., ATP, rather than the electrochemical proton gradient, most likely drives the uptake of oligopeptides.

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